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Selective down-regulation of cell surface cAMP-binding sites and cAMP-induced responses in *Dictyostelium discoideum*

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Extracellular cAMP induces an intracellular accumulation of cAMP and cGMP levels in *Dictyostelium discoideum*. cAMP is detected by cell-surface receptors which are composed of a class of fast-dissociating sites ($t_{1/2}$ = 1–2 s) and a class of slow-dissociating sites ($t_{1/2}$ = 15–150 s). Exposure of *D. discoideum* cells to 1 mM cAMP for 30 min induces a reduction of cAMP binding (down-regulation; Klein, C. and Juliani, M.H. (1977) Cell 10, 329–335). The number of fast-dissociating sites was reduced by 80–90% in down-regulated cells. These sites are composed of two forms with high and low affinity which interconvert during the binding reaction. In down-regulated cells this transition still occurred in the residual sites. The accumulation of cellular cAMP levels induced by a saturating stimulus decreased by 80–90%. The number of slow-dissociating sites was not significantly reduced in down-regulated cells, but their affinity decreased about 10-fold. The accumulation of cellular cGMP levels induced by a saturating stimulus was not decreased; however, about 20-fold higher cAMP concentrations were required to induce the same response. These results demonstrate that the cAMP transduction pathways to adenylate cyclase and guanylate cyclase are down-regulated differently. Furthermore, the results suggest that the fast-dissociating sites are involved in the activation of adenylate cyclase, while the slow-dissociating sites are coupled to guanylate cyclase.

Introduction

In the cellular slime mold, *Dictyostelium discoideum*, extracellular cAMP induces chemotaxis and aggregation, which leads to the formation of a multicellular structure. cAMP is detected by cell surface receptors, resulting in several intracellular responses such as the activation of guanylate and adenylate cyclase [1–4]. The fast increase of cGMP is supposed to be involved in the chemotactic reaction, while the produced cAMP is secreted and the cAMP signal relayed.

Recent results suggest that cAMP-binding activity of *D. discoideum* cells is heterogeneous with respect to kinetic properties [5]. The majority of the cAMP receptors release bound cAMP very fast with a half-life of 1–2 s. The class of fast-dissociating receptors is composed of high- and low-affinity binding sites (designated H and L, respectively). During the binding reaction there is a time- and cAMP-dose-dependent transition of H to L. This transition is promoted in membranes by guanine nucleotides with a specificity which suggests that it is caused by an interaction with a guanine nucleotide regulatory protein (G-protein, [6]). The transition in cells is specifically inhibited by extracellular Ca^{2+} at low concentrations (20 μM); at the same low Ca^{2+} concentration the cAMP-induced activation of adenylate cyclase is inhibited [7]. This suggests that the fast-dissociating sites are coupled via a G-protein to adenylate cyclase.

A second class of cAMP receptors is slow-disso-

ciating, and is composed of at least two types which interconvert during the binding reaction. These sites release bound cAMP with a half-life of 15 and 150 s, respectively (designated S and SS, respectively) (Ref. 8 and unpublished observations). Extracellular Ca^{2+} alters the affinity of these sites at high concentrations (400 μM). At the same Ca^{2+} concentration, the kinetics of the cAMP-induced cGMP response are altered; furthermore, these sites and the cGMP response are affected by Mg^{2+} and Mn^{2+} at, respectively, 5- and 50-fold higher concentrations than Ca^{2+} [7]. The slow-dissociating sites also interact with a G-protein [6,9]. These data suggest that the slow-dissociating sites are coupled via a G-protein to guanylate cyclase.

In many transduction pathways of hormone-like signals, the continuous presence of the hormone induces desensitization, which may be the result of several possible changes: (i) down-regulation of receptors, i.e., the hormone does not bind any longer to the receptor (the receptor might be internalized or still be exposed at the cells surface); (ii) the hormone still binds to the receptor, but the coupling between receptor and the second messenger generating enzyme (e.g. adenylate cyclase, guanylate cyclase, phospholipase C) is disconnected; (iii) the second messenger is produced by the hormone, but is not effective.

In *D. discoideum*, cAMP induces the fast adaptation of guanylate cyclase (seconds, [10,11]) and adenylate cyclase (1–2 min [12,13]). The mechanism of adaptation is not yet completely known; however, it has been shown that it belongs to the second category, i.e., between receptor and the cyclases [14,15]. cAMP also induces down-regulation of cAMP-binding. This process is relatively slow (5–30 min) and requires high cAMP concentrations [16,17].

Down-regulation in *D. discoideum* reflects a loss in the number of binding sites. However, it is not known whether the different classes of binding sites are diminished to the same extent. Moreover, it is not known whether the cAMP-induced responses are altered in down-regulated cells. In this report it is shown that exposure of *D. discoideum* cells to 1 mM cAMP during 30 min results in a 80–90% loss of the fast-dissociating binding sites. The remaining fast-dissociating sites have normal affinities and interconversions, and still couple to adenylate cyclase. The number of slow-dissociating sites is not decreased significantly, but their affinity is decreased about 10-fold. In down-regulated cells the cAMP accumulation induced by a saturating stimulus is decreased by 80–90%; the cGMP response still reaches the same value, but 20-times higher cAMP concentrations are required.

Materials and Methods

Materials. [2,8-^{3}H]cAMP (1.5 TBq/mmol), the cAMP binding protein assay kit and the cGMP radioimmunoassay kit were purchased from Amersham International, (Bucks, U.K.); cAMP and dithiothreitol were from Sigma, and 2′-deoxyadenosine 3′,5′-monophosphate (dcAMP) was from Boehringer; silicon oil AR 200 and AR 20 were obtained from Wacker Chemie, München (F.R.G.).

Culture conditions. D. discoideum cells (NC_4H) were grown in association with *Escherichia coli* 281 on a solid medium containing 3.3 g of peptone, 3.3 g of glucose, 4.5 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 2H_2O$ and 15 g of agar. Cells were harvested in the late log-phase with 10 mM sodium/potassium phosphate buffer, pH 6.5 (phosphate buffer) and freed from bacteria by repeated centrifugation at 100 × *g* for 4 min.

Down-regulation. Cells were starved for 4.5 h by shaking in phosphate buffer at a density of 10^7 cells per ml. Then the cell suspension was divided into two portions; to one portion cAMP (final concentration 1 mM) was added, the other portion was taken as control and the cells were shaken for another 30 min. After that the cells were collected, washed twice and resuspended in phosphate buffer.

cAMP-binding assay [5,8]. Cells were resuspended at a density of 10^8 cells/ml. cAMP binding was measured at 20°C in a total volume of 100 μl containing phosphate buffer, 5 mM dithiothreitol, different concentrations [^{3}H]cAMP and 80 μl cell suspension. At the times indicated, bound and free [^{3}H]cAMP were separated by centrifugating the cells through 200 μl silicon oil (AR 200/AR 20 = 1/2) in a swing-out rotor at 10000 × *g* for 15 s. Nonspecific binding was measured by including 0.1 mM cAMP in the incubation mixture and was

subtracted from all data.

cAMP relay response [18]. Cells were resuspended at a density of $6.25 \cdot 10^7$ cells/ml. Cells (80 μl) were stimulated with 20 μl of a mixture containing phosphate buffer, 5 mM dithiothreitol and 5 μM dcAMP (final concentration). At the times indicated, the reaction was stopped by the addition of 100 μl of perchloric acid 3.5% (v/v). Lysates were neutralized with 50 μl $KHCO_3$ (50% saturated), and centrifuged at $8000 \times g$ for 2 min. The cAMP content in the supernatant was measured, using the cAMP binding protein assay kit.

cGMP response [10]. Cells were resuspended at a density of $6.25 \cdot 10^7$ cells/ml. Cells (80 μl) were stimulated with 20 μl cAMP (different concentrations). At the times indicated, cells were lysed with 100 μl perchloric acid. Lysates were neutralized as described above and the cGMP concentration was measured by radioimmunoassay.

Results

In a preliminary experiment, the cAMP and cGMP accumulation induced by a saturating stimulus was measured in cells exposed to 1 mM cAMP for 30 min. Equilibrium binding at 2 nM [^{3}H]cAMP was reduced by 85% in down-regulated cells. The cAMP response was also reduced by 85%, but the cGMP accumulation was similar in down-regulated and control cells. Before performing detailed experiment on the heterogeneity of the transduction pathway, it is important to know the time interval that cells remain down-regulated after the high cAMP concentration has been removed.

Recovery from down-regulation

The time-dependent recovery, with regard to control cells, of both cell surface cAMP binding and the cAMP-induced cAMP response (cAMP relay), is shown in Fig. 1. The moment at which the cells were resuspended, after the washing procedure, is taken as time zero. For cAMP binding, cells are incubated with 2 nM [^{3}H]cAMP until equilibrium (45 s). cAMP relay was measured at 3 min after stimulation with 5 μM dcAMP. During the first 5 min, recovery is reduced, probably because the cells are unstable after the washing procedure. After that time, binding as well as cAMP relay remain constant for 20 min at a level of only 10–15% of control cells, and recovery increases at $t = 30$ min. Recovery from down-regulation shows similar kinetics for cAMP binding and for cAMP relay (Fig. 1).

From these experiments it is clear that all experiments with down-regulated cells should be performed within 20 min (5–25 min after resuspension in buffer). This short time period limits the extent of the experiments. Down-regulated cells have much lower levels of cAMP-binding and cAMP-induced responses which further complicate the measurements.

cAMP-binding (nonequilibrium kinetics)

The association of 30 nM [^{3}H]cAMP to *D. discoideum* cells is shown in Fig. 2. Maximal binding is obtained at about 6 s and then decreases, until an apparent equilibrium is reached at about 45 s. Values for down-regulated cells are given at 6 and 45 s. No differences are found in binding ratios at 6 and 45 s between control- and down-regulated cells; for both conditions the ratio is 1.5. The amount of cAMP bound, however, is about 4-fold less in down-regulated cells than in control cells. The decrease of cAMP binding between 6

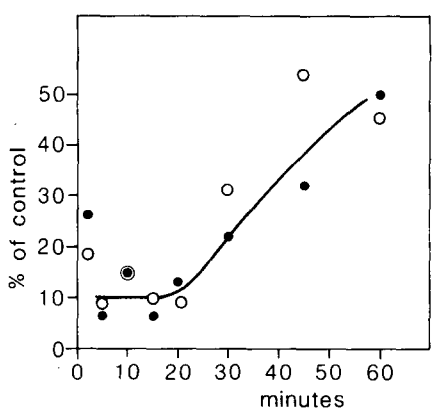

Fig. 1. *D. discoideum* cells were incubated with 1 mM cAMP for 0.5 h, centrifuged, washed twice and resuspended in phosphate buffer at 20°C ($t = 0$ min). Control cells were not exposed to 1 mM cAMP. Air was bubbled through the suspensions at a rate of about 15 ml air/min per ml. cAMP binding (●) was measured at 2 nM [^{3}H]cAMP after an incubation period of 45 s. The cAMP accumulation (○) was measured at 3 min after addition of 5 μM dcAMP with 5 mM dithiothreitol.

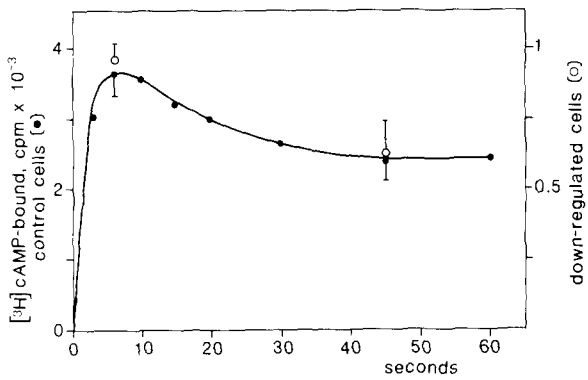

Fig. 2. Association of 30 nM [^{3}H]cAMP. Control cells (●), and down-regulated cells (○) were incubated at 20°C with 30 nM [^{3}H]cAMP and 5 mM dithiothreitol. At the times indicated the cells were centrifuged through silicon oil [5]. Data at 6 and 45 s are the means and S.D. of quadruplicate incubations; other data are the means of duplicate incubations. Nonspecific binding was measured by including 0.1 mM cAMP in the incubation mixture; its value was 300 cpm/sample and was subtracted from the data shown. The amount of cAMP bound is about 4-fold less in down-regulated than in control cells, but the relative decreases in binding between 6 and 45 s are identical.

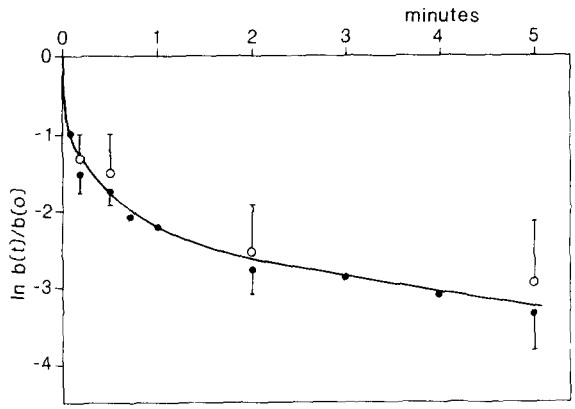

Fig. 3. Dissociation of bound cAMP. Control cells (●) and down-regulated cells (○) were preincubated with 2 nM [^{3}H]cAMP for 45 s in a volume of 100 µl. Then, at $t = 0$, 1 ml of a mixture containing phosphate buffer, 0.1 mM cAMP and 5 mM dithiothreitol was added tot he cells, and residual binding of [^{3}H]cAMP was detected at the times indicated. b(t) is the specific binding at time t; b(o) is the specific binding before the cAMP chase and was 359 cpm/sample for control cells, and 50 cpm/sample for down-regulated cells. Non-specific binding was 7 cpm/sample for both cell types. The data shown are the means and S.D. after logarithmic transformation. Data with error bars were from quadruplicate incubations; other data are from duplicate incubations.

and 45 s represents the transition of high-affinity to low-affinity binding [5]. Apparently, this transition is still present in down-regulated cells.

The dissociation of cAMP-receptor complex after equilibration with 2 nM [^{3}H]cAMP is shown in Fig. 3. In control cells, bound [^{3}H]cAMP dissociates from at least three components with different off rates: 67% with $t_{\frac{1}{2}} = 1.5$ s, 24% with $t_{\frac{1}{2}} = 15$ s (S-sites), and 9% with $t_{\frac{1}{2}} = 150$ s (SS-sites) [8]. The dissociation of cAMP in down-regulated cells is also multiphasic, with approximately the same proportioning of the binding components and about the same rate constants of dissociation. However, the amount of cAMP bound to these sites at 2 nM cAMP is about 7-fold less in down-regulated cells than in control cells.

cAMP-binding (equilibrium kinetics)

Cells were incubated with different concentrations [^{3}H]cAMP. Binding was detected either at equilibrium (sum of all binding types), or at 10 s after a chase with excess cAMP (by which time all [^{3}H]cAMP bound to the fast-dissociating sites has been released).

Binding of cAMP to all sites is shown in Fig.

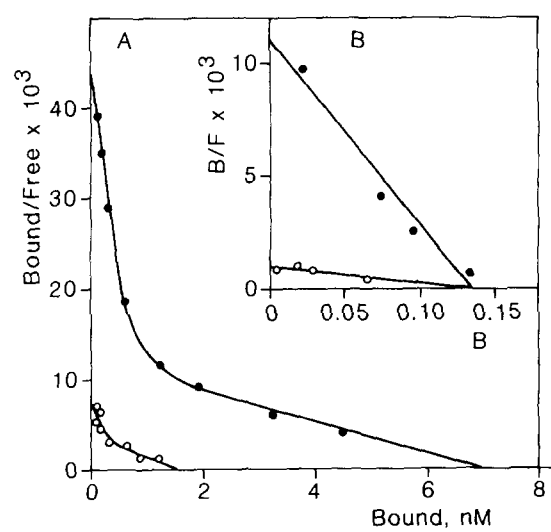

Fig. 4. Scatchard plots of fast- and slow-dissociating sites (A) and of only slow-dissociating sites (B). Control cells (●) or down-regulated cells (○) were incubated with different concentrations of [^{3}H]cAMP during 45 s. Cells were centrifuged through the silicon oil at the times indicated (A), or at 10 s after the addition of 0.1 mM cAMP; after these 10 s all [^{3}H]cAMP bound to the fast-dissociating sites has been released.

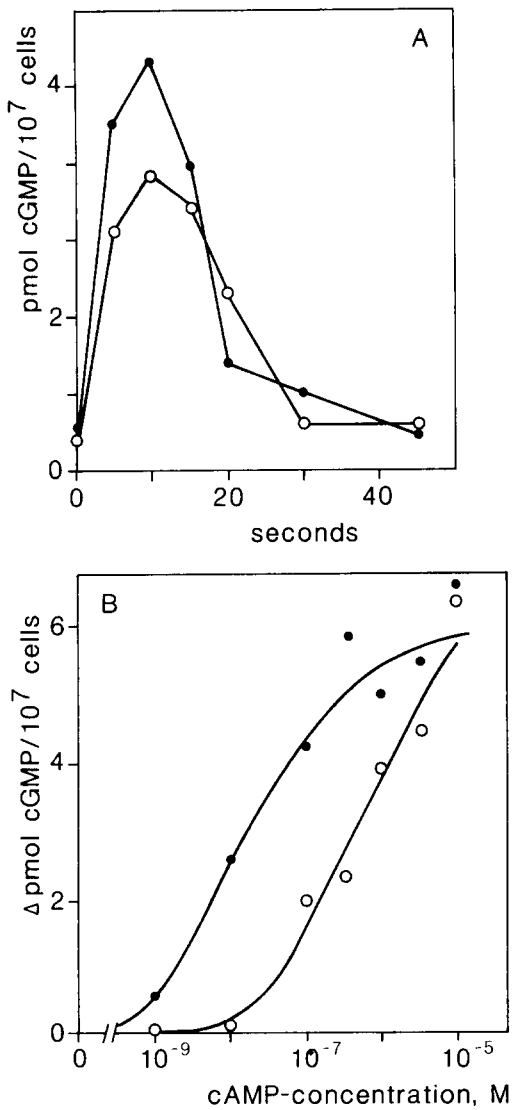

Fig. 5. cAMP-induced accumulation of cGMP levels. (A) Control cells (●) or down-regulated cells (○) were stimulated with 1 μM cAMP at $t = 0$ s. Cells were lysed at the times indicated, and the cGMP content was measured by radioimmunoassay. (B) Cells were stimulated with different cAMP concentrations at $t = 0$, and the cGMP content was measured after 10 s. The increase in cGMP above basal levels is shown. The symbols are the same as in A.

4A. The number of binding sites is decreased about 80% in down-regulated cells. The curves are non-linear in both case, and run approximately parallel. Binding of cAMP to the slow-dissociating sites is shown in Fig. 4B. The number of binding sites is not reduced significantly in down-regulated

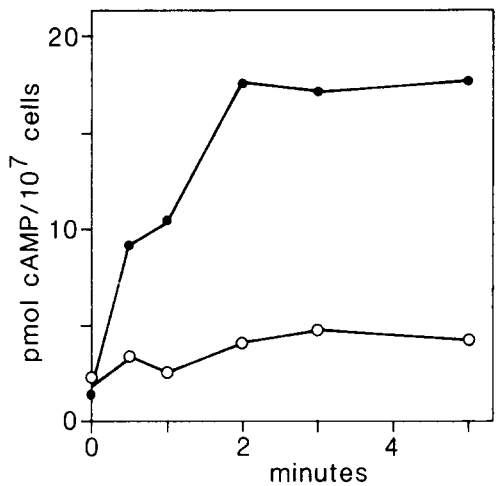

Fig. 6. dcAMP-induced accumulation of cAMP levels. Control cells (●) or down-regulated cells (○) were stimulated with 5 μM dcAMP and 5 mM dithiothreitol at $t = 0$. Cells were lysed at the times indicated, and the cAMP content was measured by isotope dilution assay.

cells. The affinity however, is reduced strongly. In control cells the apparent K_d of S- and SS-sites is 12–15 nM (Ref. 5, and unpublished observations), while in down-regulated cells $K_d = 120$ nM.

The observation that the number of slow-dissociating sites is not affected while the number of all sites is strongly reduced implies that only the fast-dissociating sites are down-regulated by the cAMP concentration.

cAMP-induced cGMP response

As stated before, down-regulated cells exhibit an almost normal cGMP accumulation in response to a saturating cAMP stimulus (1 μM). The kinetics of the response are also similar to those in control cells (Fig. 5A), with maximal cGMP levels at 10 s after stimulation and a return to basal levels after about 30 s. The dose-response curve of the cAMP-induced cGMP accumulation is shown in Fig. 5B. The maximal response is similar in control cells and in down-regulated cells. In contrast, the concentration which induces a half-maximal response is shifted from 15 nM in control cells to about 300 nM in down-regulated cells.

The dcAMP-induced cAMP response

D. discoideum cells were stimulated with 5 μM dcAMP. This analog is only 5–10-fold less active

than cAMP for surface receptors (Ref. 19, and unpublished data on the different binding types), and the concentration used is about 100-fold higher than the apparent K_a for the cAMP relay response in control cells (P.N. Devreotes, personal communication). cAMP levels were measured after stimulation with dcAMP by isotope dilution assay, using a binding protein with very low affinity for dcAMP [18]. The accumulation of cAMP reaches a maximal value after about 2–3 min in both control and down-regulated cells; the maximal response in down-regulated cells is only 10–15% of control cells (Fig. 6). With this method it is not possible to obtain accurate dose-response curves, because the secreted cAMP will stimulate the cells. However, the concentration of dcAMP used is 100-fold higher than its K_a in control cells which strongly suggests that in this experiment the maximal response is measured in control cells and in down-regulated cells.

Discussion

The cAMP-binding activity of *D. discoideum* cells is heterogeneous; fast and slow-dissociating sites have been observed [5]. These cells respond to cAMP with an increase of intracellular cAMP and cGMP levels. Long-term exposure of *D. discoideum* cells to high cAMP concentrations results in a reduction of the cAMP-binding activity and of cAMP-induced responses. (i) The release of bound cAMP has similar kinetics as in control cells (Fig. 3), which suggests that the rate constants of dissociation are not altered. (ii) The number of fast-dissociating sites is decreased by 80–90% (Fig. 4). In control cells these sites can have high and low affinity for cAMP. The proportioning of H- and L-sites changes during the binding reaction [5]. The fast-dissociating sites which remain after down-regulation are still composed of H- and L-sites, and the transition takes place as in control cells (Fig. 2). This suggests that the majority of the fast-dissociating sites do not bind cAMP any longer, but that the binding activity which remains may function normally. (iii) The number of slow-dissociating sites is decreased only slightly. Thus, exposure of cells to high cAMP concentrations does not induce down-regulation of the S- and SS-sites. Nevertheless, these sites are affected by the high cAMP concentration, because their affinity is reduced about 10-fold (Fig. 4B). (iv) Addition of a saturating stimulus to down-regulated cells yields an 80–90% inhibition of the cAMP accumulation (Fig. 6). In contrast, the maximal cGMP accumulation is not diminished in down-regulated cells, but a 20-fold higher cAMP concentration is required to induce the same response (Fig. 5).

It thus appears that exposure of *D. discoideum* cells to high cAMP concentrations induces down-regulation of the fast-dissociating sites and of the cAMP accumulation. The slow-dissociating sites and the cGMP accumulation are not down-regulated, but have a 10–20-fold decreased sensitivity to cAMP.

It is concluded that the fast-dissociating sites are coupled to adenylate cyclase, while the slow-dissociating sites are coupled to guanylate cyclase. The same conclusion was reached recently in a study of the effects of extracellular Ca^{2+} on cAMP binding and cAMP-induced responses [7]. The signal transduction pathway contains guanine nucleotide regulatory proteins [6,9,20–22]. The fast- and the slow-dissociating sites are affected by guanyl nucleotides [6,9], which suggests that the activation of both adenylate and guanylate cyclase is mediated by a G-protein. Further experiments on the interaction of fast and slow sites with G-proteins may elucidate the mechanism of excitation and adaptation of adenylate and guanylate cyclase, respectively.

Acknowledgments

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